

Supplemental Materials:

Supplemental Table 1: In silico cross-reactivity assessment of N1 and N2 primer pairs across a variety of common respiratory pathogens.

Organism	Taxonomic ID	qPCR primer 2019 nCoV_N1 BLAST match	qPCR primer 2019 nCoV_N2 BLAST match
Coronavirus SARS-CoV-2, COVID-19	2697049	100	100
Coronavirus SARS-CoV	694009	≤79	≤79
Coronavirus HKU1	290028	≤79	≤79
Coronavirus NL63	277944	≤79	≤79
Coronavirus 229E	11137	≤79	≤79
Coronavirus OC43	31631	≤79	≤79
Middle East respiratory syndrome-related coronavirus (MERS)	1335626	≤79	≤79
Adenovirus (e.g. C1 Ad.71)	10506	≤79	≤79
Human Metapneumovirus (hMPV)	162145	≤79	≤79
Influenza A	11320	≤79	≤79
Influenza B	11520	≤79	≤79
Enterovirus (e.g., EV68)	12059	≤79	≤79
Parainfluenza Virus 1 (Respirovirus 1)	12730	≤79	≤79
Parainfluenza Virus 2 (Rubulavirus 2)	1979160	≤79	≤79
Parainfluenza Virus 3 (Respirovirus 3)	11216	≤79	≤79
Parainfluenza Virus 4 (Rubulavirus 4)	1979161	≤79	≤79
RSV (Human orthopneumovirus)	11250	≤79	≤79
Rhinovirus A	147711	≤79	≤79
Rhinovirus B	147712	≤79	≤79
Rhinovirus C	463676	≤79	≤79
<i>Chlamydia pneumoniae</i>	83558	≤79	≤79
<i>Haemophilus influenzae</i>	727	≤79	≤79
<i>Legionella pneumophila</i>	446	≤79	≤79
<i>Mycobacterium tuberculosis</i>	1773	≤79	≤79
<i>Streptococcus pneumoniae</i>	1313	≤79	≤79
<i>Streptococcus pyogenes</i>	1314	≤79	≤79
<i>Bordetella pertussis</i>	520	≤79	≤79
<i>Mycoplasma pneumoniae</i>	2104	≤79	≤79
<i>Pneumocystis jirovecii</i> (PJP)	42068	≤79	≤79
<i>Candida albicans</i>	5476	≤79	≤79
<i>Pseudomonas aeruginosa</i>	287	≤79	≤79
<i>Staphylococcus epidermidis</i>	1282	≤79	≤79
<i>Streptococcus salivarius</i>	1304	≤79	≤79

Supplemental Table 2. PCR cycling conditions used by the CDC compared to the LDT PCR cycling conditions with a different thermocycler. The CDC SARS-CoV-2 protocol used the ABI 7500FastDx and called for a 2-minute incubation with uracil-DNA N-glycosylase (UNG) at 25°C. The lowest programmable temperature of our instrument (cobas z480, Roche) was 37°C. Since the UNG is active over a relatively broad temperature range, we substituted the 2-minute UNG incubation at 25°C for a 5-minute incubation at ambient room temperature.

	CDC cycling conditions (ABI 7500FastDx)		LDT cycling conditions (Roche cobas z480)	
UNG Incubation	25°C	2 mins	20-23°C	5 mins
RT Incubation	50°C	15 mins	50°C	15 mins
Enzyme Activation	95°C	2 mins	95°C	2 mins
Amplification	95°C	3 secs	95°C	3 secs
(45 cycles)	55°C	30 secs	55°C	30 secs

Appendix 1: Detailed description of discrepancy between CDC reported limit of detection and MGH limit of detection:

Possible explanations include: (1) matrix differences (the CDC used a A549 lung adenocarcinoma cell line in UTM, compared to our use of pooled clinical samples); (2) possible quantitation differences and dilutional error stacking associated with our custom-made transcribed RNA stock; or (3) differences in PCR instrumentation and cycling speeds. We observed a slightly lower efficiency of the N2-detection reagents compared to the efficiency reported in the CDC package insert. We found an approximately four-cycle lag with the N2 primer set compared to the N1 set, while results from the CDC indicated that the difference should be closer to one cycle. A collaborating institution later revealed that the N2-set performance could be rescued by extending the melt time to 5-10 seconds on instruments not capable of fast cycling. Nevertheless, we proceeded under the expectation that the process and LOD differences would not be clinically significant.

Supplemental Table 3: Assay performance in COVID-negative samples and in-vitro assessment of cross-reactivity.

COVID-negative Sample Number	Clinical results for additional respiratory pathogens	2019 nCov_N1 Ct	2019 nCov_N2 Ct	RNAse P Ct	Result	MA DPH Result	Concordance
COVID-negative 1	Negative ¹	--	--	24.67	Negative		Yes
COVID-negative 2	Negative ¹	--	--	22.77	Negative		Yes
COVID-negative 3	Negative ¹	-- ³	--	25.33	Negative		Yes
COVID-negative 4	Negative ¹	--	--	25.23	Negative		Yes
COVID-negative 5	Negative ¹	--	--	26.64	Negative		Yes
COVID-negative 6	Negative ¹	--	--	24.57	Negative		Yes
COVID-negative 7	Negative ¹	--	--	26.68	Negative		Yes
COVID-negative 8	Negative ¹	--	--	26.64	Negative		Yes
COVID-negative 9	Negative ¹	--	--	22.21	Negative		Yes
COVID-negative 10	Negative ¹	--	--	24.81	Negative		Yes
COVID-negative 11	Negative ¹	--	--	25.68	Negative		Yes
COVID-negative 12	Negative ¹	--	--	23.85	Negative		Yes
COVID-negative 13	Negative ¹	--	--	24.64	Negative		Yes
COVID-negative 14	Negative ¹	--	--	30.5	Negative		Yes
COVID-negative 15	Negative ¹	--	--	25.75	Negative		Yes
COVID-negative 16	Human Metapneumovirus	--	--	26.13	Negative		Yes
COVID-negative 17	Adenovirus, Coronavirus 229E	--	--	27.34	Negative		Yes
COVID-negative 18	Adenovirus	--	--	25.96	Negative		Yes
COVID-negative 19	Adenovirus	--	--	27.3	Negative		Yes
COVID-negative 20	Parainfluenza Virus 3	--	--	27.99	Negative		Yes
COVID-negative 21	Parainfluenza Virus 3	--	--	23.84	Negative		Yes
COVID-negative 22	Parainfluenza Virus 2	--	--	32.95	Negative		Yes
COVID-negative 23	Respiratory Syncytial Virus	--	--	24.84	Negative		Yes
COVID-negative 24	Respiratory Syncytial Virus	--	--	23.22	Negative		Yes
COVID-negative 25	Influenza B	--	--	32.95	Negative		Yes
COVID-negative 26	Influenza B	--	--	24.12	Negative		Yes
COVID-negative 27	influenza A/H1-2009	--	--	22.41	Negative		Yes
COVID-negative 28	influenza A/H1-2010	--	--	24.93	Negative		Yes
COVID-negative 29	influenza A/H1-2011	--	--	29.53	Negative		Yes
COVID-negative 30	Negative ²	--	--	23.99	Negative	Negative ⁴	Yes

¹ Negative by a multiplexed PCR for influenza A, influenza B, and RSV (Cepheid) and direct immunofluorescence antigen testing for adenovirus, human metapneumovirus, and parainfluenza types 1-3 (Light Diagnostics).

² Negative by a multiplexed PCR for influenza A, influenza B, and RSV (Cepheid).

³ This nasopharyngeal specimen originally yielded a positive result (N1 Ct 34.93, no amplification with N2, and RNAse P Ct of 25.33). The same extracted RNA was re-tested in duplicate; one reaction also gave an N1 Ct of 34.88 and the second reaction did not have amplification of N1. A new aliquot of the original NP sample was obtained, re-extracted, and re-PCR'd, which had no amplification of N1 or N2, and an RNAse P Ct of 27.52. Thus, the sample was determined to be negative.

⁴ A dedicated nasopharyngeal (NP) specimen and oropharyngeal (OP) specimen from this patient were sent to the State Lab for testing, where the NP specimen tested positive (N1 Ct of 32.05, N2 Ct of 31.40), and the OP specimen was negative. A different NP specimen collected on the same day (for flu/RSV testing) was tested by the in-house LDT assay and did not amplify with the N1 or N2 primer/probe sets, while the RNAse P Ct was 23.99. The same extracted RNA was re-tested in-house and again did not amplify with N1 or N2, while the RNAse P Ct was 24.79. Finally, the in-house NP specimen that consistently tested negative was sent to the State Lab for re-testing, and the State Lab also found that the specimen was negative for COVID-19. Thus, we believe there was a pre-analytic error, e.g. sample mislabeling, that led to an erroneous initial result of COVID-19 positivity.

1 Supplemental Table 4: Assay performance in COVID-positive clinical samples.

COVID-positive Sample Number	Clinical results for additional respiratory pathogens	2019 nCov_N1 Ct	2019 nCov_N2 Ct	RNAse P Ct	Result	MA DPH Result	Concordance
COVID-positive 1	Negative ¹	31.8	22.84	24.37	Positive	Positive	Yes
COVID-positive 2	Negative ¹	16.59	18.69	24.73	Positive	Positive	Yes
COVID-positive 3	Negative ¹	17.8	20.45	23.43	Positive	Positive	Yes
COVID-positive 4	Negative ¹	13.96	15.26	23.54	Positive	Positive	Yes
COVID-positive 5	Negative ¹	17.84	19.66	24.27	Positive	Positive	Yes
COVID-positive 6	Negative ¹	16.25	18.4	29.15	Positive	Positive	Yes
COVID-positive 7	Negative ¹	23.57	26.74	26.24	Positive	Positive	Yes
COVID-positive 8	Negative ¹	21.97	24.12	28.5	Positive	Positive	Yes
COVID-positive 9	Negative ¹	25.6	29.49	23.5	Positive	Positive	Yes
COVID-positive 10	Negative ¹	10.64	12.25	24.46	Positive	Positive	Yes
COVID-positive 11	Negative ¹	19.83	22.63	24.93	Positive	Positive	Yes
COVID-positive 12	Negative ¹	15.5	17.3	24.42	Positive	Positive	Yes
COVID-positive 13	Negative ¹	21.17	23.73	23.9	Positive	Positive	Yes
COVID-positive 14	Negative ¹	18.76	20.56	24.67	Positive	Positive	Yes
COVID-positive 15	Negative ¹	27.31	31.65	24.92	Positive	Positive	Yes
COVID-positive 16	Negative ¹	18.76	22.1	26.97	Positive	Positive	Yes
COVID-positive 17	Negative ¹	23.5	28.41	27.21	Positive	Positive	Yes
COVID-positive 18	Negative ¹	22.36	25.72	23.85	Positive	Positive	Yes
COVID-positive 19	Negative ¹	24.17	27.67	25.92	Positive	Positive	Yes
COVID-positive 20	Negative ¹	23.39	26.51	26.51	Positive	Positive	Yes
COVID-positive 21	Negative ¹	21.62	23.7	28.52	Positive	Positive	Yes
COVID-positive 22	Negative ¹	26.39	30.25	25.13	Positive	Positive	Yes
COVID-positive 23	Negative ¹	15.7	17.98	25.93	Positive	Positive	Yes
COVID-positive 24	Negative ¹	14.8	16.27	24.27	Positive	Positive	Yes
COVID-positive 25	Negative ¹	17.7	19.46	24.46	Positive	Positive	Yes
COVID-positive 26	Negative ¹	16.73	18.48	23.8	Positive	Positive	Yes
COVID-positive 27	Negative ¹	22.26	24.61	32.95	Positive	Positive	Yes
COVID-positive 28	Negative ¹	21.98	24.89	24.39	Positive	Positive	Yes
COVID-positive 29	Negative ¹	14.55	17.08	25.11	Positive	Positive	Yes
COVID-positive 30	Negative ¹	19.51	22.25	22.51	Positive	Positive	Yes

¹ Negative by a multiplexed PCR for influenza A, influenza B, and RSV (Cepheid).

2